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# The use of lacZ-transduced tumor cells enhances the sensitivity of micrometastasis detection: A comparative study of gemcitabine treatment efficacy in the mouse LM8 osteosarcoma cell model

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## Abstract

In osteosarcoma patients as well as in preclinical osteosarcoma animal models post-therapy detection of residual disease and of metastases in particular remains a great challenge. The therapeutic efficacy is often overestimated because disseminated tumor cells frequently persist undetectable as dormant micrometastases. This can be avoided in preclinical studies by tagging the tumor cells with reporter genes that allow their selective detection in normal tissue down to the single cell level. In the present study we made use of a lacZ reporter gene and reinvestigated the therapeutic effect of gemcitabine on subcutaneous primary tumor growth and metastasis of mouse LM8 osteosarcoma cells in syngeneic C3H mice. Furthermore we compared the sensitivity of LM8-lacZ and of non-transduced LM8 cells to gemcitabine *in vitro* and *in vivo* because it was recently demonstrated that expression of a GFP reporter gene in osteosarcoma cells altered their aggressiveness. The present study showed that, in contrast to previous reports, gemcitabine treatment did not completely eradicate metastasis although it efficiently suppressed the growth of primary tumors and macrometastases. The results also showed that minimal residual disease is not restricted to the lungs, but also occurs in the liver and the kidneys. The direct comparison of the LM8-lacZ with the LM8 cells furthermore demonstrated that constitutive expression of the lacZ reporter gene has no effect on the aggressiveness of the cells or their sensitivity to gemcitabine. The LM8-lacZ cell-derived osteosarcoma mouse model thus represents a highly sensitive and reliable model for evaluation of anticancer drug efficacy *in vivo*.

***Keywords:*** osteosarcoma; metastasis detection; gemcitabine; lacZ; mouse model; macrometastases; lung cancer; liver cancer; kidney cancer

## Introduction

The detection of metastases remains one of the predominant challenges in clinical care of cancer patients as well as in preclinical cancer research models. Importantly, when the disseminated tumor cells remain dormant as single cell metastases or small cell clusters they may escape detection by currently established diagnostic methods such as computer tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET). In preclinical cancer research, this problem has been largely solved by tagging the tumor cells with specific reporter genes encoding for example green (GFP), yellow (YFP) or red (dsRed) fluorescent protein, the bioluminescence enzyme luciferase or the bacterial enzyme beta-galactosidase (beta-gal). An advantage of luciferase and the fluorescence proteins is their applicability *in vivo* to monitor tumor development and metastasis over time. In contrast, the bacterial beta-gal, encoded by the lacZ gene of the bacterial lac-operon, still provides the highest *ex vivo*-resolution, allowing the detection of metastases down to the single cell level

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[1]. A potential problem of stably introduced reporter genes is their possible influence on the cell phenotype ranging from reduced viability to increased growth and metastatic potential. Dass and Choong, for example, showed that stable transfection of SaOS-2 osteosarcoma (OS) cells with GFP leads to loss of alkaline phosphatase (ALP) and osteoprotegerin (OPG) expression *in vitro* and more aggressive primary tumor growth and enhanced metastatic dissemination *in vivo* [2]. To our knowledge, such an interference with the metabolic activity of tumor cells has not been described for the lacZ reporter gene, and an earlier study with lacZ-tagged Dunn cells and the highly metastatic subline LM8 did not reveal any indication for an influence of lacZ on the cell phenotype [1]. However, in a subsequent drug testing study with the potentially anti-neoplastic active antibiotic taurolidine the treatment groups in the LM8 (-lacZ) and the K7M2 (-lacZ) syngeneic OS mouse models showed an unexpected and significantly increased metastatic load in lungs and livers compared to the vehicle control treated mice [3]. This raises the question of whether the expression of the reporter gene lacZ may have a specific influence on the sensitivity of the OS cells towards anti-neoplastic drugs. In the present study, we therefore tested the potential influence of the lacZ reporter gene on the anti-tumor and anti-metastatic activity of gemcitabine.

Gemcitabine is widely used in the treatment of solid cancers such as pancreatic [4], bladder [5], breast [6], ovarian [7], cervical [8], and non-small-cell lung cancer (NSCLC) [9], whereas results with OS have been less impressive [10, 11]. However, recent work has shown that in combination with drugs such as docetaxel [12], the bisphosphonate zoledronic acid [13], or the flavonoid genistein [14], gemcitabine works synergistically with beneficial effects.

Gemcitabine (2´,2´-difluoro 2´deoxycytidine) is an analog of deoxycytidine and therefore acts as nucleoside metabolic inhibitor in DNA replication [15]. Gemcitabine is administered as a pro-drug and requires not only cellular uptake by nucleoside transporters but also intracellular activation by initial phosphorylation and subsequent conversion into the active metabolites [16]. The metabolites possess several intracellular activities, including inhibition of DNA polymerase, incorporation into the DNA resulting in termination of chain elongation, prevention of DNA repair, and inhibition of ribonucleotide reductase [15, 16]. Due to this complex intracellular activation process and the multiple inhibitory functions gemcitabine was an optimal candidate to study the potential influence of beta-gal overexpression on the drug sensitivity of the LM8 OS cells. Furthermore, gemcitabine has already been successfully tested in the LM8 OS mouse model but without any reporter gene [17]. This gave us the opportunity to compare our results not only with the internal "lacZ-free" control LM8 cells but also with already published data. In addition, for optimal comparability we followed exactly the experimental protocol of Ando et al. [17].

## Materials and methods

### *Cell culture and reagents*

The mouse osteosarcoma cell line LM8 [18] was provided by Takafumi Ueda (Osaka University Graduate School of Medicine, Osaka, Japan). The LM8 cells were stably transduced with a lacZ reporter gene and cultured as recently reported [1]. Gemcitabine (GEMZAR®) was purchased from Eli Lilly (Suisse) S.A.

### *Cytotoxicity assay*

5 x $10^3$ LM8 or LM8-lacZ cells per well were seeded in 96-well plates and allowed to adhere overnight. The cells were then incubated in triplicates with increasing concentrations of gemcitabine (0.1 – 1000 nM) or with vehicle alone for 48h. Following drug treatment, the cells were incubated for 3h with 10 $\mu$l/well of WST-1 reagent (Roche, Switzerland) and the cell viability was assessed as described [19]. Prism 4 network software was used to calculate the half-maximal growth inhibitory concentration (IC50) of the drug. The experiments were repeated three times.

### *Subcutaneous osteosarcoma mouse model*

Female 7-8 week old C3H/HeNCrl mice (20 g average body weight) were obtained from Charles River Laboratories (Sulzfeld, Germany) at least 10 days prior to initiation of experimental treatments. Housing conditions and experimental protocols were in accordance with the guidelines of the Swiss Federal Veterinary Office (FVO) and approved by the authorities of the canton of Zurich. On day 0, $10^7$ LM8 control or lacZ-transduced LM8 (LM8-lacZ) cells in 300 $\mu$l PBS were injected subcutaneously into the right flank of the syngeneic C3H mice (20 mice per cell line). Primary tumor growth to a measurable size (measured with a caliper and determined as length x width$^2$/2) was allowed to proceed for 7 days prior to treatment. Mice were then separated into groups of 10 animals with a similar distribution of tumor sizes. Intraperitoneal treatment of the mice with 200 $\mu$l vehicle (0.9% NaCl solution) or 150 mg/kg body weight gemcitabine was started immediately after primary tumor staging and grouping of the mice and was performed once per week until the end of the study. The health of the mice was monitored daily and the tumor size was measured once a week. On day 26, the mice were sacrificed under anesthesia and the lungs perfused with PBS and then fixed under inflation with 3% paraformaldehyde as described [1]. From all mice, the left lobe of the lungs, as well as the two upper and the middle lobes of the livers and both kidneys were stained with the chromogenic beta-gal substrate 5-Bromo-4-chloro-3-indolyl-$\beta$-D-galactoside

(X-Gal) following a published protocol [20] and the lacZ expressing metastases quantified as described below.

*Quantification of LacZ expressing metastases on the organ surfaces*

Photographs of dissected lungs, livers and kidneys were taken with a Kappa PS 20 C digital camera (Kappa Opto-Electronics GmbH) connected to an OpMi-1 binocular microscope (Zeiss) and imported as TIF files into Power Point® software. Macrometastases on organ surfaces, defined as indigo-blue stained foci >0.1 mm in diameter, were counted. Close-ups were taken with the same camera but connected to an Eclipse E600 microscope (Nikon Corporation) and micrometastases (<0.1 mm diameter) were counted in 10 randomly selected close-ups of 1 $mm^2$ organ surface.

*Quantification of metastases in H&E stained paraffin sections*

Formaldehyde fixed tissue samples from the lungs and livers of all mice were embedded in paraffin and 10 $\mu$m sections were cut on a microtome, deparaffinized, and stained with hematoxyline and eosine (H&E). For each mouse, macrometastases (>0.1 mm) and micrometastases (<0.1 mm) were counted in lung and liver sections under an Eclipse E600 microscope equipped with a 10x objective and calibrated beforehand with an objective micrometer ruler (Nikon Corporation).

*Statistical analysis*

Results are presented as the mean ± standard error of the mean (SEM) of original data or as % of control. Statistical analysis was performed with the GraphPad Prism® 5.01 software. Normally distributed (Kolmogorov-Smirnov test) unpaired data sets were analyzed with the two-tailed unpaired t-test, and other unpaired data with the two-tailed Mann-Whitney rank sum test. Normally distributed paired data were analyzed with the two-tailed paired t-test and other paired data with the two-tailed Wilcoxon signed rank test. Data were considered significantly different for P values <0.05.

## Results

*Sensitivity of lacZ-tagged and untagged LM8 cells to gemcitabine in vitro*

To determine whether lacZ-transduced and non-transduced LM8 cells show any difference in sensitivity to gemcitabine, we first analyzed the dose-dependent cytotoxicity in a standard WST-1 cell viability assay. The two cell lines were equally sensitive to gemcitabine treatment, with a mean IC50 of 7.5 nM for LM8 (range: 4.4-10.1 nM) and 7.2 nM for LM8-lacZ cells (range: 5.6-10.0 nM) after 24 h of treatment (data not shown). A treatment of 48 h decreased the mean IC50 of gemcitabine to 3.2 nM for LM8 (range: 0.9-6.4 nM) and to 3.2 nM for LM8-lacZ cells (range: 0.8-5.9 nM) (Figure 1).

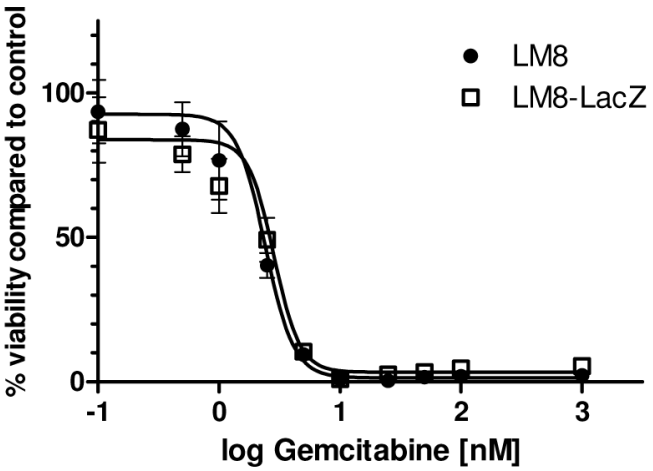


**Figure 1** Comparison of gemcitabine cytotoxicity on LM8 and LM8-lacZ cells *in vitro*. Gemcitabine dose-dependent cell viability was determined in a WST-1 assay as described in materials and methods. Representative dose-response curves out of 3 independent experiments are shown. Each data point represents the mean value of measurements done in triplicates.

*Gemcitabine treatment efficiently prevents the growth of LM8 and LM8-lacZ cell-derived primary tumors*

We next compared non-transduced with the lacZ-transduced LM8 cells in their ability to grow to primary tumors and spontaneous metastases when injected subcutaneously into syngeneic C3H mice. In addition, we assessed in both models the tumor growth and metastasis suppressive efficacy of intraperitoneal administered gemcitabine. The tumor growth in the control (saline-injected) animals progressed rapidly in a logarithmic fashion and at an equal rate regardless of whether the tumors were derived from LM8 or LM8-lacZ cells (lines with unfilled and filled triangles in Figure 2B), leading within 4 weeks to large subcutaneous primary tumors with a diameter of more than 10 mm (left image in Figure 2A). In contrast, intraperitoneal treatment with a weekly dose of 150 mg/kg gemcitabine, starting on day 7, dramatically inhibited the growth of primary tumors at the injection site (right image in Figure 2A). The gemcitabine treatment was comparably efficient on both the growth of LM8- and of LM8-lacZ-derived tumors (lines with unfilled and filled circles in Figure 2B) and essentially prevented any further tumor progression following the first application on day 7.

*Gemcitabine treatment significantly inhibits LM8-lacZ metastasis to lung, liver and kidney*

We next took advantage of a recently developed protocol for improved visualization of metastases, combining the sensitive colorimetric detection of lacZ-tagged cells in tissues with a technique of in situ organ perfusion and fixation [20]. This allowed us to quantitatively analyze the formation of lung, liver and kidney metastases on whole mounts of organs of the LM8-lacZ injected mice, and to evaluate the efficacy of gemcitabine treatment on the development of both macro- (>0.1 mm in diameter) and micro- (<0.1 mm in diameter) metastases (Figure 3). The top panels in Figure 3A-C show representative lungs,

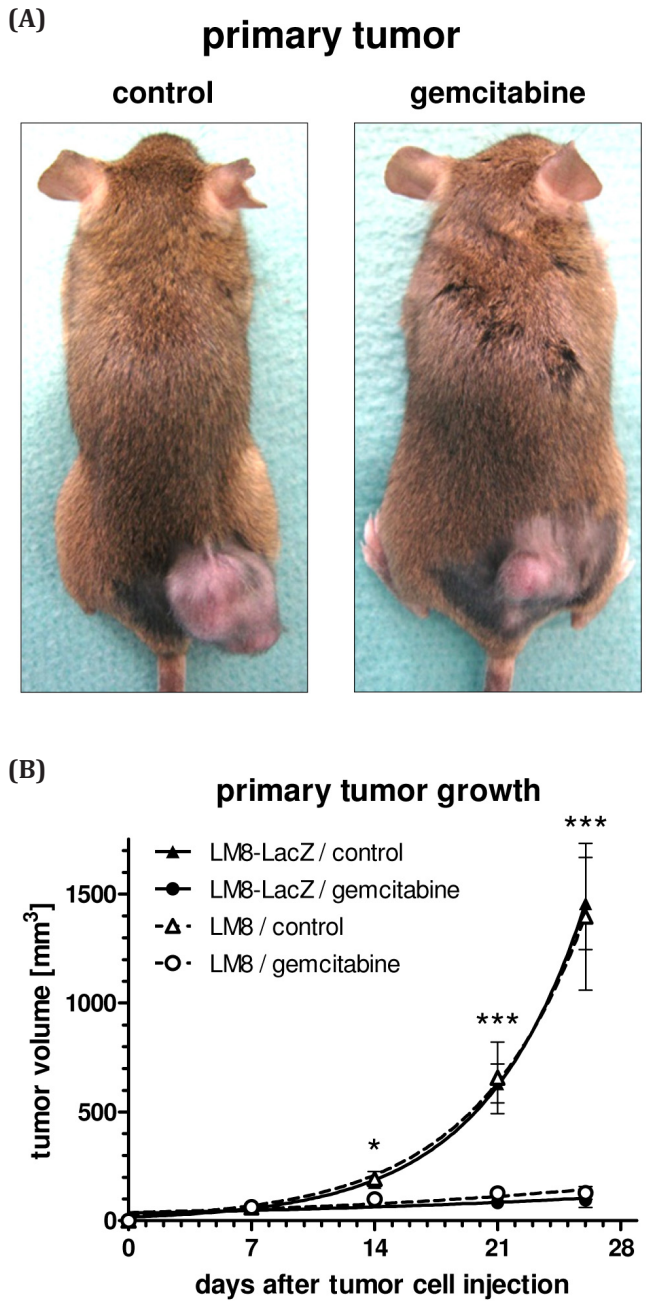


**Figure 2** Comparison of subcutaneous primary tumor growth in mice injected with LM8 or LM8-lacZ cells and treated with gemcitabine or vehicle control. Treatment was started after tumor staging on day 7 post tumor cell injection. A. Images of a representative non-treated and a gemcitabine treated mouse 26 days after injection of LM8 cells, B. Mean (± SEM) tumor growth over time in mice (n=10 per group) injected with tumor cells and treated as indicated. *P<0.05, ***P<0.001 compared to the respective control.

livers and kidneys of control and of gemcitabine treated mice and illustrate the sensitive detectability of lacZ expressing metastatic cells against the pale background of the perfused organ tissue. In the bottom panels of Figure 3 the quantitative effect of gemcitabine treatment on the formation of metastases in the lungs, livers and kidneys of mice is shown in comparison to the metastatic burden of vehicle treated control animals. The data revealed that the gemcitabine treatment regimen (weekly dose of 150 mg/kg body weight for 3 weeks) almost completely abolished the formation of macrometastases, with over 99% reduction in the lungs (Figure 3A bottom left panel) and a complete prevention of macroscopic foci in the livers and kidneys (Figure 3B and C bottom left panels). However, the high sensitivity of our tumor cell detection technique revealed that micrometastatic lesions were not completely eradicated by gemcitabine even at the rather high dose used in the present study. The drug reduced the number of micrometastases in the lungs and kidneys to approximately 10% of that observed in untreated animals (Figure 3A and C, bottom right panels). In the livers, however, the number of micrometastases was only reduced to approximately 45% of that seen in control animals (Figure 3B, bottom right panel).

*Histological comparison of gemcitabine treatment efficacy on metastasis of LM8 and LM8-lacZ cell-derived tumors*

To evaluate the inhibitory efficacy of gemcitabine treatment on metastasis formation in mice subcutaneously injected with non-transduced LM8 cells in comparison to the therapeutic effect in LM8-lacZ cell-bearing mice, H&E stained lung and liver sections of control saline- (images i and iii in Figure 4A and B) and of gemcitabine treated (images ii and iv in Figure 4A and B) animals were histologically examined. The quantification of macro- and micrometastases in lungs and livers (Figure 4C-F) revealed a comparable anti-metastatic effect of gemcitabine treatment on LM8 and LM8-lacZ cells. Lung sections of gemcitabine treated mice in the LM8 group displayed less than 4% of the number of macrometastases detected in lung sections of vehicle treated control animals and macrometastases remained undetectable in gemcitabine treated animals of the LM8-lacZ group (Figure 4C).The number of lung micrometastases was also similarly (87% and 96%) reduced in gemcitabine treated mice of the LM8 and LM8-lacZ group compared to respective controls (Figure 4D). In the liver sections of both groups no macrometastases could be detected in gemcitabine treated mice reflecting 100% inhibition compared to the corresponding control groups (Figure 4E). However, while micrometastases were also completely absent in liver sections of gemcitabine treated mice injected with LM8-lacZ cells, the respective gemcitabine treated mice in the LM8 group showed only 50% reduction of liver micrometastases compared to the control (Figure 4F).

## Discussion

Metastasis is a major predictor of poor prognosis in OS. The early and sensitive detection of metastases thus represents an important goal of monitoring the progression of the disease and assessing the efficacy of treatment. Using lacZ-transduced highly metastatic mouse LM8 OS cells and a recently developed method for improved detection of metastases in the lung and liver, we here report the effects of high dose (150 mg/kg) gemcitabine on primary tumor growth and metastasis in a well-established mouse model of OS. We chose LM8 cells and the C3H syngeneic

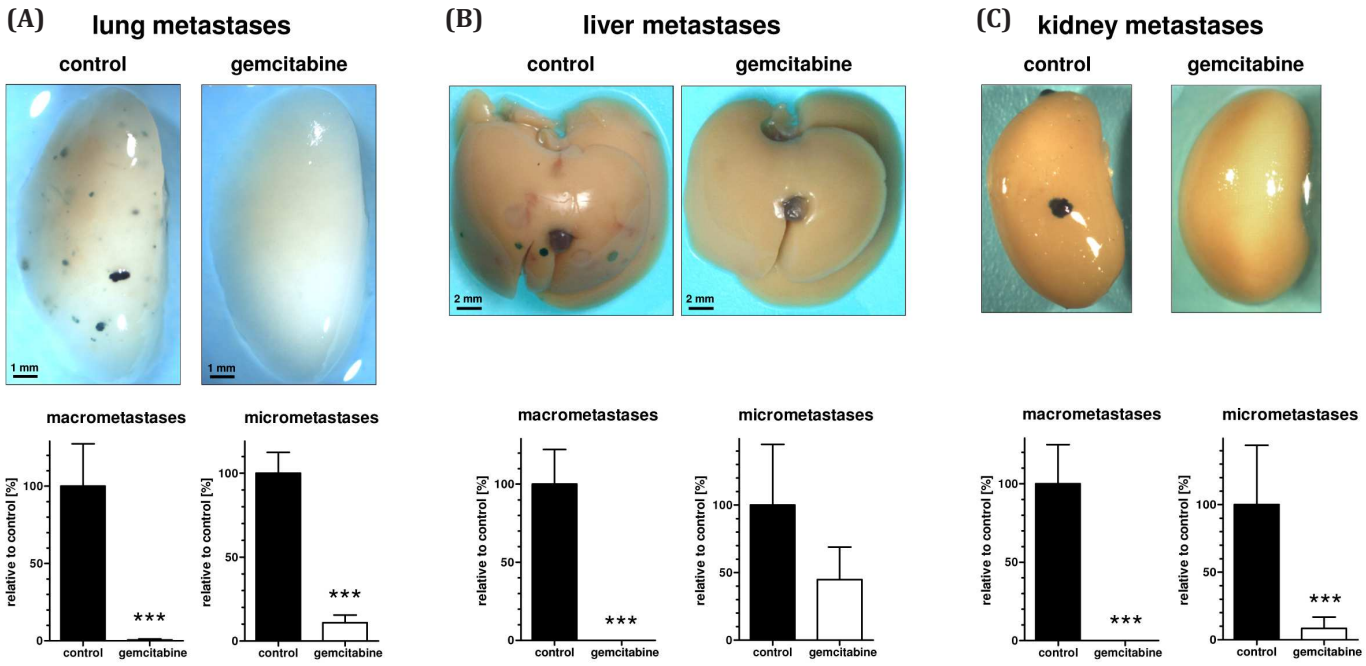


**Figure 3** Inhibition by gemcitabine of spontaneous metastasis of LM8-lacZ cells to lung, liver and kidney. Representative images of whole mounts of X-gal stained metastases-bearing lungs (A), livers (B) and kidneys (C) of vehicle- or gemcitabine treated mice injected with LM8-lacZ cells (top panels), and quantitative analysis (bottom panels) of relative mean (± SEM) numbers (in percent of respective controls) of X-gal stained macrometastases (>0.1 mm) and micrometastases (<0.1 mm) on lung (A), liver (B) and kidney (C) surfaces of vehicle- (control) or gemcitabine treated mice (n=10 per group). Mean (± SEM) numbers of metastases per organ in control mice were 90.6 ± 24.7 lung macrometastases, 17.7 ± 2.2 lung micrometastases, 18.5 ± 4.1 liver macrometastases, 0.3 ± 0.1 liver micrometastases, 4.8 ± 1.2 kidney macrometastases, 3.9 ± 1.7 kidney micrometastases. ***P<0.001 compared to the respective control.

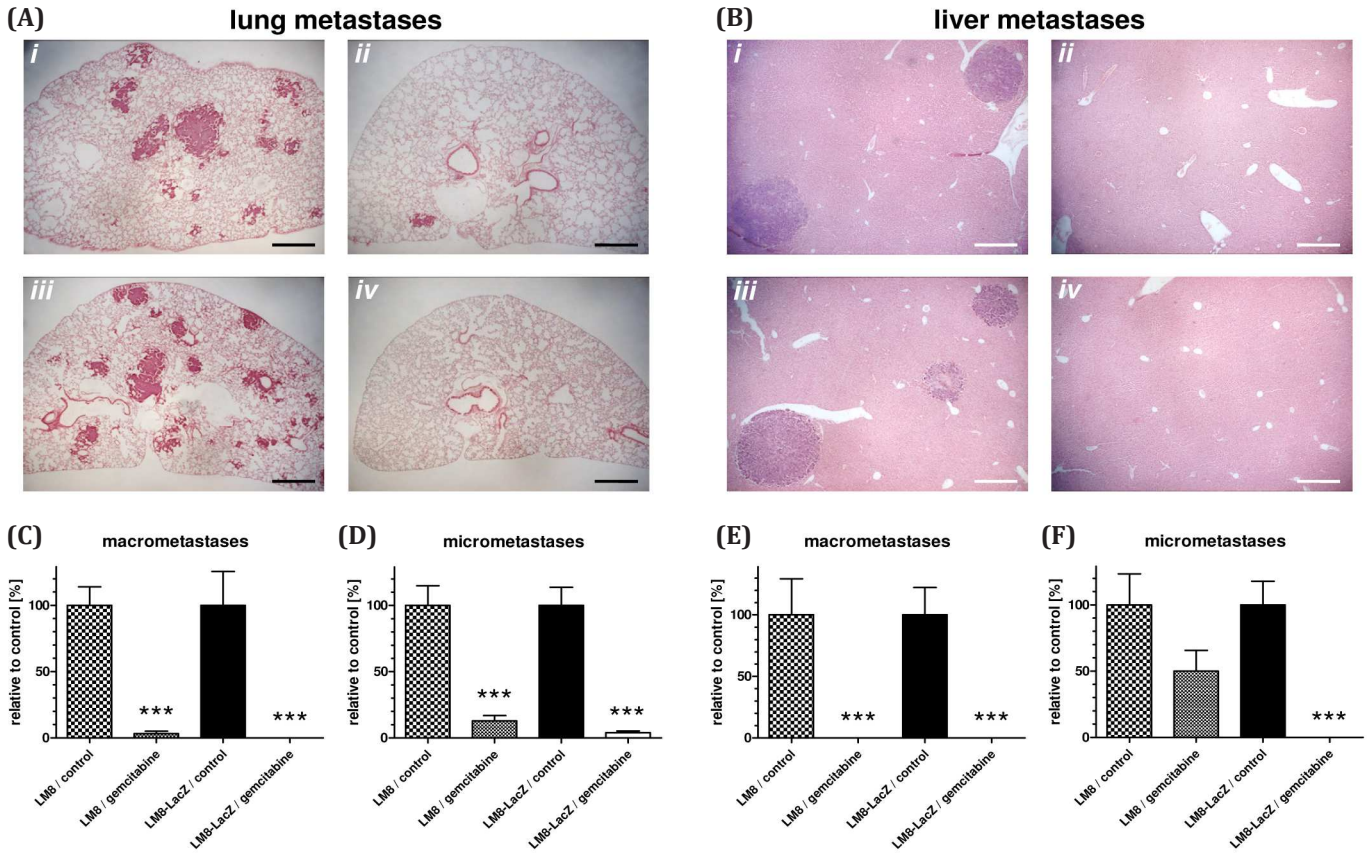


**Figure 4** Quantification of macro- and micrometastases in H&E stained tissue sections of lung and liver lobes. The upper panels show representative images of lung (A) and liver (B) sections of mice injected with LM8 (i-ii) or LM8-lacZ cells (iii-iv) and treated with vehicle control (i, iii) or gemcitabine (ii, iv). The lower panels (C-F) show relative mean (± SEM) numbers per H&E stained tissue section (in percent of respective controls) of lung macro- (C) and micrometastases (D) as well as of liver macro- (E) and micrometastases (F). Mean (± SEM) numbers of metastases per tissue section in control mice were for LM8 6.4 ± 0.9 lung macrometastases, 10.5 ± 1.6 lung micrometastases, 1.2 ± 0.4 liver macrometastases, 1.4 ± 0.3 liver micrometastases, and for LM8-lacZ 15.4 ± 3.9 lung macrometastases, 17.8 ± 2.4 lung micrometastases, 5.3 ± 1.2 liver macrometastases, 1.5 ± 0.3 liver micrometastases. ***P<0.001 compared to the respective control.

mouse model of subcutaneous tumor cell inoculation as used by Ando et al. [17] to demonstrate the improved sensitivity of metastasis detection with lacZ-transduced cells, the visualization of otherwise non-detectable micrometastases and to verify that lacZ-transduction did not affect the drug sensitivity of osteosarcoma cells.

The validity and distinct superiority of the tumor cell detection system based on LacZ expression was highlighted by direct comparison of the data obtained from counting X-gal stained (indigo-blue) metastatic foci on organ whole mounts with the corresponding results of the histological analysis in organ sections. Both examination techniques revealed comparable results for the therapeutic effect of gemcitabine, but the analysis with lacZ-transduced tumor cells enabled a more precise quantification: for example, while in one mouse of the gemcitabine group macrometastases were clearly detectable on a lung lobe upon X-gal staining, the subsequent histological examination of the same lobe failed to detect them. Consequently, the ability of the lacZ/X-gal system to detect malignant lesions at the single cell level on whole mounts of organs enables a better quantitative assessment of therapeutic success, as well as a more accurate detection of residual or recurring micrometastatic cancer lesions over time, notably without the need for tedious tissue fixation, paraffin embedding, sectioning, and staining.

We also investigated the potential influence of the constitutive expression of beta-galactosidase on the therapeutic efficacy of the gemcitabine treatment because it was recently shown that stable expression of GFP as cell-tracking marker can alter the tumor-promoting properties of OS cells [2]. Here, *in vitro* gemcitabine cytotoxicity experiments demonstrated that the stable transduction of LM8 cells with the lacZ gene did not alter their sensitivity towards the chemotherapeutic drug. Both cell lines, LM8-lacZ and non-transduced LM8, were equally sensitive to the cytotoxicity of gemcitabine with virtually no cells remaining viable after treatment with 10 nM gemcitabine for 48 h. Thus, these cells were more sensitive than many human OS cell lines including SaOS, HuO9 and MG63 [17, 21], for which IC50 values of 100 nM or higher were reported, and concentrations of gemcitabine above 500 nM were needed to completely suppress cell viability [14, 17]. In *in vivo* experiments with mice injected with LM8 or LM8-lacZ cells, measurements over time of subcutaneous primary tumor growth at the injection site demonstrated that the stable transduction of the LM8 cells with the lacZ gene neither altered their potential to induce rapidly growing tumors, nor the sensitivity of these tumors towards gemcitabine treatment. The constitutive beta-galactosidase expression in the LM8-lacZ cells also did not influence the metastasis suppressive potency of gemcitabine. The comparative evaluation of the metastatic load in H&E stained tissue sections revealed a similar reduction of lung and liver macrometastases and lung micrometastases in both groups upon gemcitabine treatment. Only the residual number of liver micrometastases was different in LM8- and LM8-lacZ injected mice; however, this discrepancy is most likely related to the limitation of the histological analysis and the overall low number of liver micrometastases.

The comparison of the presented data with the results of the study by Ando and co-workers [17] revealed an additional interesting and important finding. While we confirmed the almost complete suppression of primary tumor growth and the virtual absence of macrometastases in the lung when the mice were treated with gemcitabine, we found, in contrast to Ando et al. [17], a clear residual micrometastatic burden in the lungs. Furthermore, we detected a residual metastatic disease in livers and kidneys upon gemcitabine treatment, which was neither reported in the study of Ando et al. [17] nor in the study of Koshkina and co-workers who administered gemcitabine as an aerosol [22]. Thus, while gemcitabine treatment very efficiently inhibits primary tumor growth and prevents the formation of macrometastases in lungs, livers and kidneys, it cannot completely eradicate the micrometastatic burden in the LM8 OS mouse model.

Today it is well established that the sensitivity of tumors, which do not well respond to a certain chemotherapeutic agent, can be increased by a combination treatment with another drug. For example, human U2OS cells were found to be partially resistant to gemcitabine *in vitro* at concentrations up to 50 $\mu$M, but became more sensitive to the drug in combination with genistein [14]. A new aspect of the present study is that, although the tumor cells responded very well to gemcitabine treatment *in vitro*, a minimal residual disease, consisting of disseminated single cells or small cell clusters, remained detectable *in vivo* in 70% of the mice after the therapy. This apparent gemcitabine-resistance was likely brought about by a non-proliferating state of dormancy of the tumor cells in the host tissue [23]. Thus, the data of the present study not only underline the importance of improving the sensitivity and resolution of micrometastasis detection in experimental *in vivo* tumor models, they also justify the current discussion about the implications of metastatic dormancy on the success of cancer treatment [23] and show the need to address this biological phenomenon in future therapy concepts.

## Conclusion

Our research indicates that osteosarcoma patients who are well responding to the chemotherapeutic treatment might be afterwards still at high risk of an undetected minimal residual disease and later recurrence. Consequently, improving the sensitivity and resolution of micrometastasis detection is of utmost importance to avoid an overestimation of the therapeutic success.

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**Conflict of interest**

The authors wish to express that they have no conflict of interest.

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